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Sirtuin-mediated deacetylation pathway stabilizes Werner syndrome protein

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Abstract **Caloric restriction (CR) is known to promote longevity in various species. Sirtuin-mediated deacetylation has been shown to be related to the promotion of longevity in some species. Here, we show that CR of rats led to an increase in the level of Werner syndrome protein (WRN), a recognized DNA repair protein. In addition, CR simultaneously increased the level of SIRT1, a mammalian sirtuin. In HEK293T cells, sirtuin inhibitors decreased the WRN level, and this effect was suppressed by proteasomal inhibitors. Furthermore, we found a decrease in the WRN level in *Sirt1*-deficient mice. These results indicate that sirtuin-mediated deacetylation stabilizes WRN.**

Structured summary:
MINT-6603869:
ubiquitin (uniprotkb:P62988) *physically interacts* (MI:0218) with *WRN* (uniprotkb:Q14191) by *pull down* (MI:0096)

Keywords: SIRT; Werner syndrome; Longevity; Caloric restriction

1. Introduction

Proteins undergo many kinds of posttranslational modifications, and these are known to modulate enzymatic activity and changes in localization and regulate protein–protein interactions. In our previous studies, we examined the sumoylation [1], ubiquitination [2], polyglutamylation [3,4], and phosphorylation [5] of proteins as posttranslational modifications. In the current study, we focused on sirtuins—nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that have been linked to aging biology. Sirtuins differ from the ligases and kinases described in our previous reports because they require NAD for reactions. The analysis of sirtuins is expected to provide knowledge on aging from another perspective.

Sirtuins, including yeast silent information regulator 2 (Sir2) and its orthologs, have been shown to exhibit NAD-dependent histone deacetylase (type III HDAC) activity in an in vitro deacetylation assay [6]. Small-molecule activators and inhibitors of sirtuins have been characterized [7]. Sirtuin activators are known to increase the lifespan of *Saccharomyces cerevisiae* [8], *Caenorhabditis elegans*, and *Drosophila melanogaster* [9], when the Sir2 enzyme is functional.

SIRT1 is the closest mammalian ortholog of yeast Sir2 [10]. It is known to target some substrates besides histones for deacetylation, such as the tumor suppressor p53 [11,12]. In mammalian cells, SIRT1 regulates apoptosis in response to some stresses. *Sirt1*-knockout mice have been generated and reported by two different groups, and the commonly recognized phenotypes in these mice are small body size and a high percentage of embryonic or postnatal lethality [13,14].

Some genetic defects in the DNA repair pathways, including DNA metabolism, have been linked to premature aging-like features in the mouse [15]. Moreover, in humans, several progeroid syndromes such as Werner syndrome are related to genetic defects in the DNA repair system [16]. Molecular defects in the DNA repair pathways have been suggested to cause genomic instability and premature aging-like symptoms, although some of these symptoms are considered to reflect normal aging [17].

Considering this background, we focused on the molecular connections between sirtuin-mediated deacetylation and DNA repair in order to study longevity and premature aging. In this paper, we show that sirtuin-mediated deacetylation stabilizes WRN. Our findings will help understand the contribution of sirtuin deacetylase activity to longevity.

2. Materials and methods

2.1. Animals

All procedures related to the care and treatment of animals were in accordance with the institutional guidelines and those lay down by the National Institute of Health and the Animal Care and Use Committee (Mitsubishi Kagaku Institute of Life Sciences). Caloric restriction of rats and tissue extraction were performed at the Material Research Center (Tokyo, Japan). Detailed information on food intake and body weight is provided in Fig. S1. *Sirt1*-heterozygous mice, designated $Sirt1^{+/\Delta}$, were generated in a previous study [13], and homozygous mutant offspring ($Sirt1^{\Delta/\Delta}$) from heterozygous parents were generated by in vitro fertilization and embryo transfer (IVF-ET).

2.2. Western blot analysis

Liver tissue was homogenized in a mixture of 20 mM Hepes (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 1% Triton X-100,

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and protease inhibitor cocktail (Roche, Basel, Switzerland), and it was then centrifuged at $20000 \times g$ for 10 min. The soluble fraction was added to sodium dodecyl sulfate (SDS) sample buffer. For harvesting the cultured cells, the samples were washed with PBS (–) and lysed with SDS sample buffer. The resulting samples (10 μg of protein) were used for immunoblot analysis.

2.3. RT-PCR analysis

Liver tissue from animals and culture cells were homogenized in Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan), and total RNA was isolated and reverse transcribed with ReverTra Ace (TOYOBO, Tokyo, Japan). Real-time PCR was conducted by ABI PRISM7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. Each data point in individual experiments was calculated from the mean of triplicate determinations. Conventional PCR was performed with AmpliTaq Gold polymerase (Applied Biosystems) in Fig. 4. Primer sequences are provided in the Supplemental methods section.

2.4. Cell culture and drug treatment

Human embryonic kidney (HEK293T) cells were cultured at 37 °C in air with 10% CO_2. Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) was used for the culture. Cells were treated with sirtinol (Sigma, St. Louis, MO, USA). Cycloheximide (Sigma), trichostatin A (Sigma), epoxomicin (Peptide Institute, Osaka Japan), and MG132 (Peptide Institute) were also used in the assay.

2.5. Affinity purification

His-ubiquitin was overexpressed in HEK293T cells, and purified using TALON affinity resin (Clontech, Mountain View, CA, USA) following lysis of the cells with phosphate buffer (0.1 M sodium phosphate buffer [pH 7.4], 0.3 M NaCl and 10 mM imidazole) containing 6 M guanidine hydrochloride. The resins were washed three times with phosphate buffer containing 8 M urea, and then the final wash was carried out with phosphate buffer.

3. Results

3.1. Increase in liver WRN content of calorie-restricted rats

In order to determine the association between sirtuins and DNA repair, we used the technique of caloric restriction (CR), which is an effective intervention for increasing the lifespan in the case of many different organisms [18]. It has been reported that the effect of CR is mediated by sirtuins in some organisms [19,20]. This implies that the level of a SIRT1-associated protein fluctuates in calorie-restricted animals. On the other hand, CR was found to increase in vivo DNA repair, as reported in an unscheduled DNA synthesis (UDS) assay [21]. Recently, Li's group reported that acetylated WRN exhibits low helicase and exonuclease activities and that SIRT1 can reverse this suppression of WRN activity via deacetylation [22]. Since the correlation between the dynamics of WRN and the deacetylase activity of sirtuin remains unclear, we examined the WRN protein levels in calorie-restricted rats (Figs. 1A and S1). The SIRT1 level in the livers of the calorie-restricted rats increased, as reported previously [23]. Notably, the WRN protein levels were also significantly increased, while those of topoisomerase I (TOPO I), which is known to interact with WRN [24], were not (Fig. 1A). Interestingly, the transcription of WRN mRNA was not observed to be increased in the calorie-restricted rats (Fig. 1B). These results show that CR posttranscriptionally increases WRN expression in animals.

3.2. Decrease in the WRN level in the HEK293T human culture cell line by SIRT1 deacetylase inhibitor

We next aimed to determine whether the increase in the WRN protein levels depended on sirtuin deacetylase activity. Sirtinol and trichostatin A (TSA) have been characterized as a sirtuin deacetylase inhibitor [25] and a type I and II HDAC inhibitor [26], respectively. Notably, treatment with sirtinol efficiently led to a reduction in the WRN level, while treatment with TSA did not (Fig. 2A), indicating that sirtuins specifically regulate the WRN protein levels. As a control, we showed that these two inhibitors induced p53 acetylation, which is consistent with the known effect of both types of inhibitors in the regulation of p53 acetylation [11]. WRN regulation appears to be posttranscriptional, as indicated by the real-time RT-PCR analysis data presented in Fig. 1B. Therefore, we decided to examine the possibility that WRN is regulated through stabilization of the protein levels. For this purpose, cycloheximide (CHX), a translational inhibitor, was used (Fig. 2B). The half life of WRN decreased in the cells treated with sirtinol (sirtinol +: 4.3 h) compared with the control (sirtinol −: more than 8 h). On the other hand, a decreased mRNA level of WRN was not observed at least in the sample treated with sirtinol for 4 h (Fig. 2C). These results indicate that sirtuin-mediated deacetylation is important for the stabilization of WRN protein.

3.3. Degradation of WRN through the 26S proteasome in presence of an SIRT1 deacetylase inhibitor

As indicated above, the dynamics of WRN protein is regulated posttranslationally. Therefore, we analyzed the effect of proteasome inhibitors on the levels of the WRN protein. The

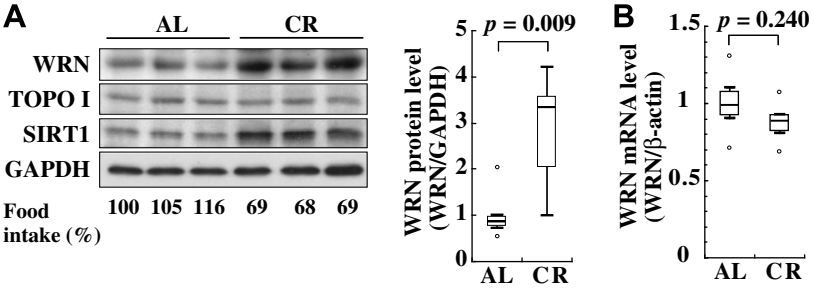

Fig. 1. Caloric restriction induces an increase in the WRN protein levels in the livers of rats. (A) Representative results of Western blot are shown in the left panels along with the relative amount of food intake. The graph on the right shows the relative values of the WRN protein calculated from the Western blot signal intensities. (B) Real-time RT-PCR was performed for a comparative analysis of WRN mRNA level between AL and CR rats. Median values are represented by the lines within the boxes. The boxes indicate 50% of the values (the 25th and 75th centiles). Circles represent outliers, which were also used to calculate the median values. AL: $n = 6$; CR: $n = 6$.

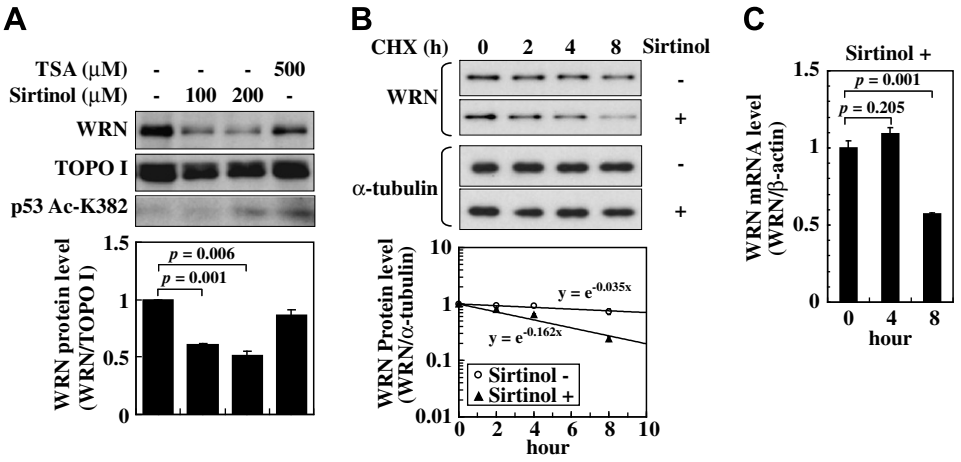

Fig. 2. Level of WRN decreases in cultured cells in the presence of a SIRT1 deacetylase inhibitor. (A) HEK293T cells were incubated with sirtinol or TSA at the indicated concentrations for 20 h. TOPO I served as a loading control. (B) HEK293T cells were treated with cycloheximide (30 μg/ml), a translational inhibitor, for the indicated time in presence or absence of sirtinol (100 μM). (C) Real-time RT-PCR was performed for a comparative analysis of WRN mRNA level in presence of sirtinol. The graphs represent the average values, and approximation curves are shown. Error bar = S.E.M. ($n = 3$).

ubiquitin–proteasome system is known selectively degrade proteins [27]. As shown in Fig. 3A, the sirtinol-induced decrease in the WRN level was suppressed by epoxomicin or MG132, both of which are specific proteasome inhibitors. Next, we performed affinity purification to His-ubiquitin-tagged proteins (Fig. 3B). Ubiquitinated WRN, detected in the high molecular region of SDS–polyacrylamide gel, was accumulated by the co-treatment of sirtinol and MG132. These results indicate that the 26S proteasome degraded WRN through the ubiquitin pathway when the sirtuin deacetylase activity was suppressed.

3.4. Decrease in the WRN level in the liver of *Sirt1*$^{\Delta/\Delta}$ mice

The results in Fig. 2 suggests the possibility that a defect in one of the sirtuins leads to decrease in the protein level of WRN. Since SIRT1 is the closest mammalian ortholog of yeast Sir2, we analyzed the dynamics of WRN in *Sirt1*$^{\Delta/\Delta}$ mice. The WRN level in the liver was lower in *Sirt1*$^{\Delta/\Delta}$ mice than in *Sirt1*$^{+/+}$ mice (Fig. 4). This decrease appears to have occurred posttranscriptionally, because the RT-PCR analysis did not show a difference in the WRN mRNA signals between the *Sirt1*$^{\Delta/\Delta}$ and *Sirt1*$^{+/+}$ mice (Fig. 4).

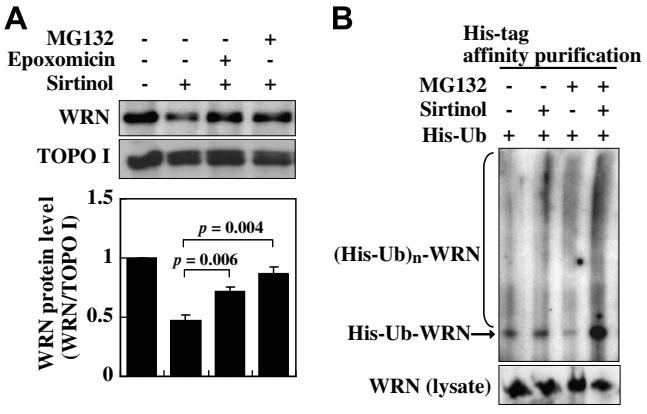

Fig. 3. WRN is degraded through the 26S proteasome in presence of an SIRT1 deacetylase inhibitor. (A) HEK293T cells were treated with epoxomicin (1 μM, 14 h) and MG132 (5 μM, 14 h), both proteasome inhibitors, in the presence of sirtinol (100 μM, 20 h). The graph represents the average values. Error bar = S.E.M. ($n = 3$). (B) His-ubiquitin was expressed in the cells in presence of sirtinol (100 μM, 8 h) or MG132 (5 μM, 8 h). After affinity purification of His-ubiquitin-tagged proteins, they were analyzed by Western blot analysis with anti-WRN antibody.

4. Discussion

In this study, we showed that the sirtuin deacetylation pathway stabilizes WRN. Defects in functional sirtuin led to a decrease in the WRN level (Fig. 2A), and this decrease was suppressed by proteasome inhibitors (Fig. 3A). Our results suggest that sirtuin deacetylase activity protects WRN from ubiquitination and sequential degradation by the 26S proteasome. Recently, it has been reported that the acetylation of WRN suppresses its helicase and exonuclease activities and that SIRT1 catalyzes the deacetylation of WRN [22]. These findings seem to be consistent with our results: both indicate that sirtuin positively regulates WRN function. Li's report showed that SIRT1 also mediates the re-entry of WRN into the nucleolus following DNA damage. Therefore, the instability of WRN demonstrated in our study may result from a delay in the translocation of WRN to the nucleolus in the absence of functional SIRT1. It seems that it is unnecessary for acetylated WRN, which is probably incompetent, to predominantly remain in the nucleoplasm, where various DNA repair and metabolic processes are underway; therefore, inefficient WRN needs to be degraded to ensure that there is no prejudicial activity. Otherwise, SIRT1 may degrade WRN irrespective of its localization and deacetylation. WRN could be ubiquitinated by a ubiquitin ligase that is deacetylated by SIRT1. The identification and characterization of such putative ubiquitin ligases will be the focus of future studies.

Quantitative analysis showed that the mRNA level of WRN was not difference between the cells treated with sirtinol for 0 and 4 h (Fig. 2C). Because the half life of WRN protein was 4.3 h in presence of sirtinol (Fig. 2B), posttranslational

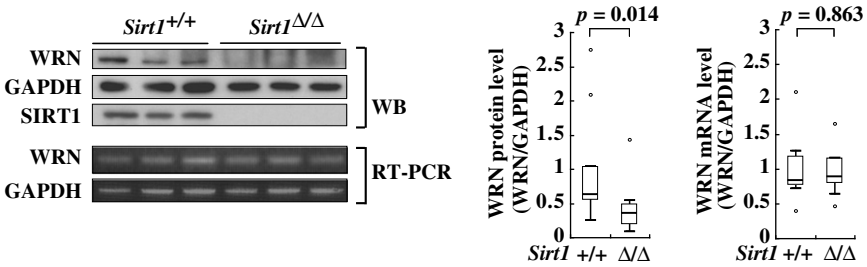

Fig. 4. WRN protein level is decreased in the livers of $Sirt1^{\Delta/\Delta}$ mice. Representative results of Western blot and RT-PCR are shown in the left panels. The graphs on the right show the relative values of the WRN protein and mRNA levels calculated from the Western blot and RT-PCR-signal intensities, respectively. Median values are represented by the lines within the boxes. The boxes indicate 50% of the values (the 25th and 75th centiles). Circles represent outliers, which were also used to calculate the median values. +/+: $n = 9$; Δ/Δ: $n = 9$.

regulation should dominantly affect the dynamics of WRN at least for that period. On the other hand, the mRNA level of WRN decreased in the cells treated with sirtinol for 8 h. This result suggests the possibility that transcriptional regulation is also involved with the decrease of WRN protein for long term.

Mice lacking SIRT6, a member of the mammalian Sir2 family, show genomic instability through the DNA base excision repair pathway and lead to aging-associated degenerative phenotypes [28]. In addition, it has been shown that SIRT6 is stabilized by nutrient deprivation and CR in cultured cells and rats, respectively [29]. While the details of the molecular interaction between SIRT6 and DNA repair are not clear, it is speculated that CR can regulate the SIRT6-mediated stability of WRN and that other sirtuins may also be implicated in this regulation.

By considering the effects of CR and the DNA repair system on lifespan and premature aging, respectively, our findings provide valuable clues for an understanding of the mechanism underlying normal aging.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.031.

References

[1] Kahyo, T., Nishida, T. and Yasuda, H. (2001) Involvement of PIAS1 in the sumoylation of tumor suppressor p53. Mol. Cell 8, 713–718.
[2] Yao, I. et al. (2007) SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. Cell 130, 943–957.
[3] Ikegami, K., Mukai, M., Tsuchida, J., Heier, R.L., Macgregor, G.R. and Setou, M. (2006) TTLL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites. J. Biol. Chem. 281, 30707–30716.
[4] Ikegami, K. et al. (2007) Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. Proc. Natl. Acad. Sci. USA 104, 3213–3218.
[5] Inoue, E. et al. (2006) SAD: a presynaptic kinase associated with synaptic vesicles and the active zone cytomatrix that regulates neurotransmitter release. Neuron 50, 261–275.
[6] Imai, S., Armstrong, C.M., Kaeberlein, M. and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403, 795–800.
[7] Grubisha, O., Smith, B.C. and Denu, J.M. (2005) Small molecule regulation of Sir2 protein deacetylases. FEBS J. 272, 4607–4616.
[8] Howitz, K.T. et al. (2003) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 425, 191–196.
[9] Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., Tatar, M. and Sinclair, D. (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430, 686–689.
[10] Frye, R.A. (1999) Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem. Biophys. Res. Commun. 260, 273–279.
[11] Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L. and Gu, W. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 107, 137–148.
[12] Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L. and Weinberg, R.A. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 107, 149–159.
[13] Cheng, H.L. et al. (2003) Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc. Natl. Acad. Sci. USA 100, 10794–10799.
[14] McBurney, M.W., Yang, X., Jardine, K., Hixon, M., Boekelheide, K., Webb, J.R., Lansdorp, P.M. and Lemieux, M. (2003) The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol. Cell. Biol. 23, 38–54.
[15] Lombard, D.B., Chua, K.F., Mostoslavsky, R., Franco, S., Gostissa, M. and Alt, F.W. (2005) DNA repair, genome stability, and aging. Cell 120, 497–512.
[16] Martin, G.M. and Oshima, J. (2000) Lessons from human progeroid syndromes. Nature 408, 263–266.
[17] Goto, M. (1997) Hierarchical deterioration of body systems in Werner's syndrome: implications for normal ageing. Mech. Ageing Dev. 98, 239–254.
[18] Weindruch, R. (1996) The retardation of aging by caloric restriction: studies in rodents and primates. Toxicol. Pathol. 24, 742–745.
[19] Guarente, L. and Kenyon, C. (2000) Genetic pathways that regulate ageing in model organisms. Nature 408, 255–262.
[20] Guarente, L. and Picard, F. (2005) Calorie restriction—the SIR2 connection. Cell 120, 473–482.
[21] Lipman, J.M., Turturro, A. and Hart, R.W. (1989) The influence of dietary restriction on DNA repair in rodents: a preliminary study. Mech. Ageing Dev. 48, 135–143.
[22] Li, K. et al. (2008) Regulation of WRN protein cellular localization and enzymatic activities by SIRT1-mediated deacetylation. J. Biol. Chem. 283, 7590–7598.
[23] Cohen, H.Y. et al. (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305, 390–392.

[24] Opresko, P.L., Cheng, W.H., von Kobbe, C., Harrigan, J.A. and Bohr, V.A. (2003) Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. Carcinogenesis 24, 791–802.
[25] Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D. and Schreiber, S.L. (2001) Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J. Biol. Chem. 276, 38837–38843.
[26] Yoshida, M., Kijima, M., Akita, M. and Beppu, T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. 265, 17174–17179.
[27] Varshavsky, A. (2005) Regulated protein degradation. Trends Biochem. Sci. 30, 283–286.
[28] Mostoslavsky, R. et al. (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124, 315–329.
[29] Kanfi, Y. et al. (2008) Regulation of SIRT6 protein levels by nutrient availability. FEBS Lett. 582, 543–548.